Contribution of Tight Junction Proteins to Ion, Macromolecule, and Water Barrier in Keratinocytes

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Tight junctions (TJs) form a selective barrier for ions, water, and macromolecules in simple epithelia. In keratinocytes and epidermis, TJs were shown to be involved in individual barrier functions. The absence of the TJ protein claudin-1 (Cldn1) in mice results in a skin-barrier defect characterized by lethal water loss. However, detailed molecular analyses of the various TJ barriers in keratinocytes and the contribution of distinct TJ proteins are missing. Herein, we discriminate TJ-dependent paracellular resistance from transcellular resistance in cultured keratinocytes using the two-path impedance spectroscopy. We demonstrate that keratinocyte TJs form a barrier for Na^+, Cl^-, and Ca^{2+}, and contribute to barrier function for water and larger molecules of different size. In addition, knockdown of Cldn1, Cldn4, occludin, and zonula occludens-1 increased paracellular permeabilities for ions and larger molecules, demonstrating that all of these TJ proteins contribute to barrier formation. Remarkably, Cldn1 and Cldn4 are not critical for TJ barrier function for water in submerged keratinocyte cultures. However, Cldn1 influences stratum corneum (SC) proteins important for SC water barrier function, and is crucial for TJ barrier formation for allergen-sized macromolecules.

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INTRODUCTION

Tight junctions (TJs) are cell–cell junctions consisting of a variety of transmembrane proteins, e.g., claudins (Cldns), occludin (Ocln), and junctional adhesion molecules, and cytosolic plaque proteins, e.g., the zonula occludens (ZO) proteins ZO-1–3 (Schneeberger and Lynch, 2004; Aijaz *et al.*, 2006). They are well known in simple epithelia to form selective paracellular barriers for ions, as well as small and large molecules (Steed *et al.*, 2010; Shen *et al.*, 2011). In the stratum granulosum of the epidermis, as well as in submerged keratinocyte cultures under high-calcium conditions, a variety of TJ proteins have been localized at the cell–cell borders, and typical TJ structures have been identified (Kitajima *et al.*, 1983; Morita *et al.*, 1998; Pummi *et al.*, 2001; Yoshida *et al.*, 2001; Brandner *et al.*, 2002; Furuse *et al.*, 2002; Brandner *et al.*, 2006; Helfrich *et al.*, 2006).

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Abbreviations: Cldn, claudin; FD4, 4 kDa FITC-dextran; FD40, 40 kDa FITC-dextran; KD, knockdown; Ocln, occludin; R, resistance; SC, stratum corneum; siRNA, small interfering RNA; TER, transepithelial resistance; TJ, tight junction; ZO, zonula occludens

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Measurements of transepithelial resistance (TER) suggest a barrier function of TJs to ions in submerged keratinocyte cultures (Helfrich *et al.*, 2006; Yuki *et al.*, 2007); however, until now, transcellular and paracellular resistance, measured by the two-path impedance spectroscopy (Krug *et al.*, 2009), was not distinguished. Lanthanum tracer experiments in skin indicate a TJ barrier for this ion in the stratum granulosum. However, lamellar body contents have also been hypothesized to form this barrier (Elias *et al.*, 1977). For other ions, the role of TJs, neither in cultured keratinocytes nor in skin, has been determined, even though it was hypothesized that TJs in the epidermis are important for the formation of a Ca^{2+}-gradient (Kurasawa *et al.*, 2011; O'Neill and Garrod, 2011; Kirschner *et al.*, 2012). The TJ barrier function for intermediate-sized molecules, and macromolecules has been shown for fluorescein (332 Da; De Benedetto *et al.*, 2011), as well as for 3, 4, and 40 kDa FITC-dextrans in cultured keratinocytes (Mertens *et al.*, 2005; Yuki *et al.*, 2007; Kirschner *et al.*, 2011), and for a 557-Da tracer in skin (Furuse *et al.*, 2002; Kirschner *et al.*, 2010b). Nevertheless, the role of different TJ proteins in forming this barrier is unclear.

The absence of Cldn1, a TJ protein localized in all layers of the epidermis, in mice, results in increased transepidermal water loss, which leads to death within the first day of birth. However, the role of TJs in water permeability of keratinocytes and the specific role of Cldn1 is unclear. In addition, although it has been proposed that reduced expression of Cldn1 in uninvolved skin of atopic dermatitis patients contributes to an increased accessibility of the skin for antigens, the role of Cldn1 for barrier function for molecules with a size of widely

distributed allergens (e.g., house dust mite/Der p1: 25 kDa; cat/Fel d1: 38 kDa) has not been investigated.

To address these open questions, we performed an extensive study investigating the ability of TJs in human primary keratinocytes to form barriers for ions in general and specifically for Na^+, Cl^-, and Ca^{2+}. Furthermore, we investigated barrier properties for water, intermediate-sized (fluorescein; 332 Da) and larger molecules (FITC-dextran; 4 and 40 kDa). Finally, we performed knockdown (KD) experiments of Cldn1, Cldn4, Ocln, and ZO-1, which are well defined in human and mouse skin and several skin diseases (Kirschner *et al.*, 2010a), to investigate the individual roles of TJ proteins.

RESULTS

Human keratinocyte TJs form a barrier to ions, intermediate-sized molecules, macromolecules, and water

TJ formation was induced in human primary keratinocytes by the elevation of extracellular Ca^{2+} levels (Ca^{2+} switch) and the TER increased with a peak at 48 hours, which declined to a steady-state value at 96 hours (Figure 1a). In cell culture monolayers, TER represents the complete resistance of an epithelium, consisting of both the paracellular resistance of the TJs (R^{para}) and the transcellular resistance of the apical and basolateral cell membranes (R^{trans}). Changes in the TER can be caused by alterations in either R^{para} or R^{trans}, or both. Therefore, to discriminate the contributions of R^{para} and R^{trans} to the changes in TER, we performed two-path impedance spectroscopy. Although there was a slight increase in R^{trans}, the increase in TER was mainly reflected by changes in R^{para} (Figure 1b). To further characterize R^{para}, we measured paracellular ion permeability and found a decrease and subsequent slight increase in the permeabilities for Na^+, Cl^-, and Ca^{2+} after Ca^{2+} switch (Figure 1c). As demonstrated by their permeability ratios, the paracellular permeability for cations was slightly higher than for anions, and for monovalent cations higher than for divalent cations (Supplementary Figure 1a online). This preference, Na^+ over Cl^- and Na^+ over Ca^{2+}, remained constant at all time points, indicating that it is independent of the strength of the barrier.

To investigate the size selectivity of paracellular permeability in human keratinocytes, the permeability for fluorescein (332 Da), 4 kDa FITC-dextran (FD4), and 40 kDa FITC-dextran (FD40) was measured (Figure 1d). After induction of TJ formation, a reduction of permeability could be seen for all three molecules. At 24 hours after Ca^{2+} switch, permeability

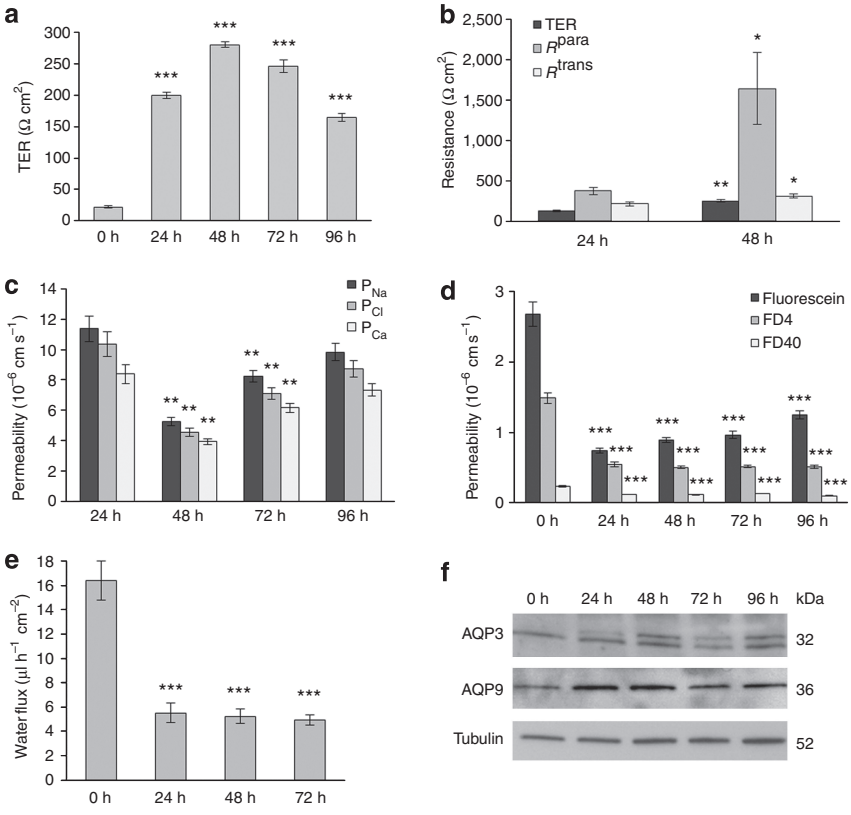

Figure 1. Tight junctions (TJs) in human keratinocytes form a barrier for ions, larger molecules, and water. (**a**) Transepithelial resistance (TER) at indicated time points after Ca^{2+} switch ($n=9$; mean ± SEM; ***$P<0.001$ compared with 0 hours). (**b**) Two-path impedance spectroscopy showing epithelial (TER), paracellular (R^{para}), and transcellular (R^{trans}) resistance ($n=4$; mean ± SEM; *$P<0.05$, **$P<0.01$, 48 hours vs. 24 hours). (**c**) Permeabilities for Na^+, Cl^-, and Ca^{2+} at indicated time points ($n=6$–8; mean ± SEM; **$P<0.01$ compared with 24 hours; values cannot be determined at 0 hours because of indefinite permeability). (**d**) Permeability for fluorescein, 4 kDa FITC-dextran (FD4), and 40 kDa FITC-dextran (FD40; $n=9$; mean ± SEM; ***$P<0.001$ for all tracers compared with 0 hours). (**e**) Water flux at indicated time points ($n=4$–5; mean ± SEM; ***$P<0.001$). (**f**) Western blot of AQP3, AQP9, and tubulin at indicated time points ($n=3$). Representative examples of experiments with individual cell lines are shown.

reduction was more pronounced for fluorescein (72 ± 2%) and FD4 (63 ± 3%) than for FD40 (48 ± 5%). Interestingly, the lowest permeability for these molecules was already achieved 24 hours after induction of TJ formation, despite the TER increasing up until 48 hours (Figure 1a). Subsequently, the permeability for fluorescein increased slightly but significantly, whereas the permeability for FD4 and FD40 remained at a steady-state level.

Further, we investigated water barrier formation. Transepithelial water flux was induced by an osmotic gradient and significantly decreased following TJ formation (Figure 1e). As the two major aquaporins of human and mouse epidermis, AQP3 and AQP9 (Brandner, 2012), were found unchanged or even upregulated (Figure 1f), this suggests that the decrease in water permeability was mainly due to a decrease in TJ-controlled paracellular water flux. Additional evidence for a paracellular water barrier is the fact that water flux in the absence of any osmotic gradient, induced by a paracellular NaCl flux, strongly decreased with paracellular barrier formation (Supplementary Figure 1b online).

To examine the correlation between barrier formation for ions, intermediate-sized molecules, and macromolecules with alterations in TJ protein expression and localization, protein expression in cultured cells was analyzed by western blot and localization by immunofluorescence (Figure 2). Cldn1, Ocln, and ZO-1 were already expressed in basal keratinocyte cultures (0 hours) without TJ formation, whereas Cldn4 was not (Figure 2a, b). After the Ca^{2+} switch induced TJ formation, there was a significant increase in Cldn1, Cldn4, Ocln, and ZO-1 at 24 hours, which decreased at later time points. Interestingly, the Ocln lower molecular weight band (~55 kDa) was predominantly expressed in low-calcium-cultured keratinocytes (0 hours), whereas higher molecular weight bands (~65–70 kDa), reflecting additional phosphorylation (Wong, 1997; Lemini-Lopez *et al.*, 2006), were induced by Ca^{2+} switch. TJ proteins were not appreciably present at the cell–cell borders under low-calcium conditions and only slight cytoplasmic staining was found. After 24 hours, all TJ proteins localized at the cell–cell borders; the same is true for later time points, although additional cytoplasmic staining increased for all proteins (Figure 2c). Notably, both Cldn1 protein and staining intensity gradually decreased.

Role of individual TJ proteins in the various TJ barrier functions

To analyze the role of distinct TJ proteins in TJ barrier function, we performed transient transfections by small interfering RNA (siRNA) and knocked down Cldn1, Cldn4, Ocln, and ZO-1 for up to 120 hours in primary human keratinocytes (see Figure 5d for Cldn1). After 48 hours, a significant reduction of protein levels for Cldn1 (83 ± 8%), for Cldn4 (46 ± 8%), for Ocln (43 ± 4%), and for ZO-1 (89 ± 5%) were observed (Supplementary Figure 2a,b online). Immunofluorescence staining and mRNA analysis (Supplementary Figure 2c,d online) confirmed the downregulation. The KD of each TJ protein resulted in a decrease of TER (Figure 3a). Decreased TER was exclusively due to a decrease in R^{para} in Cldn4 and Ocln KD cells, whereas an additional small decrease in R^{trans} was observed in Cldn1 and ZO-1 KD cells; however, there was a much more substantial decrease in R^{para} (Figure 3b). In all KD cells, an increase in ion permeability for Na^+, Cl^-, and Ca^{2+} was observed, without selectivity between the various ions (Figure 3c).

With regard to larger molecules, an increased permeability for fluorescein and FD4 was found after KD of all investigated proteins. For FD40, a significant increase in permeability was only observed for Cldn1 and ZO-1 KD cells (Figure 3d).

Because Cldns are thought to be the key players in water barrier function of TJs (Rosenthal *et al.*, 2010), we next examined water flux in Cldn1 and Cldn4 KD cells. Surprisingly, water flux measurements showed no difference between the Cldn KD cells and the corresponding controls at all time points (Figure 4a for 48 hours) and when using different siRNAs (data not shown). These data indicate that both Cldn1 and Cldn4 are dispensable for the water barrier formation of TJs in human submerged keratinocyte cultures. As this result was surprising given the mortality of Cldn1-deficient mice due to increased water loss (Furuse *et al.*, 2002), we next examined whether there might be a difference between mice and men. We analyzed water flux in keratinocytes from Cldn1-deficient mice, and just like the KD experiments we found no difference in water flux between keratinocytes of wild-type and Cldn1-deficient mice (Figure 4b), but significantly lower levels in TER 48 and 72 hours after Ca^{2+} switch in the Cldn1-deficient keratinocytes (Figure 4c). These results confirm the finding that Cldn1 is dispensable for water barrier formation of TJs.

Influence of TJ protein KD on the expression and localization of other TJ proteins

To determine whether TJ protein KD altered the expression or localization of other TJ proteins, we analyzed Cldn1, Cldn4, Ocln, and ZO-1. Western blot analysis revealed no significant changes in Cldn1, Cldn4, and Ocln KD cells, and only a slight but significant increase in Cldn1 expression in ZO-1 KD keratinocytes (Supplementary Figure 3a online). Immunolocalization in Cldn1 KD cells revealed that Cldn4 was slightly more intensely localized at the cell–cell borders, whereas Ocln localization was fragmented and ZO-1 localization was unaltered. Similarly, in Ocln KD cells Cldn4 was more intensely localized at the cell–cell borders, although no alteration was observed for Cldn1 and ZO-1. In Cldn4 KD cells, no alteration of Cldn1, Ocln, and ZO-1 localization was observed. Finally, in ZO-1 KD cells the localization patterns of Cldn1, Cldn4, and Ocln were all fragmentary at the cell–cell borders (Supplementary Figure 3b online).

Influence of Cldn1 KD on the expression of aquaporins and cornified envelope proteins

Because of the surprising result that the absence of Cldn1 had no effect on TJ water barrier, we investigated whether this might be due to a compensatory alteration of aquaporins. We did not observe any alteration of AQP3 and AQP9 levels in human Cldn1 KD cells (Figure 5a). In mouse keratinocytes, although we found no differences for AQP3, AQP9 was significantly increased under low-calcium conditions and decreased 72 hours after Ca^{2+} switch (Figure 5b,c). However, these could not explain the absence of an alteration of water flux.

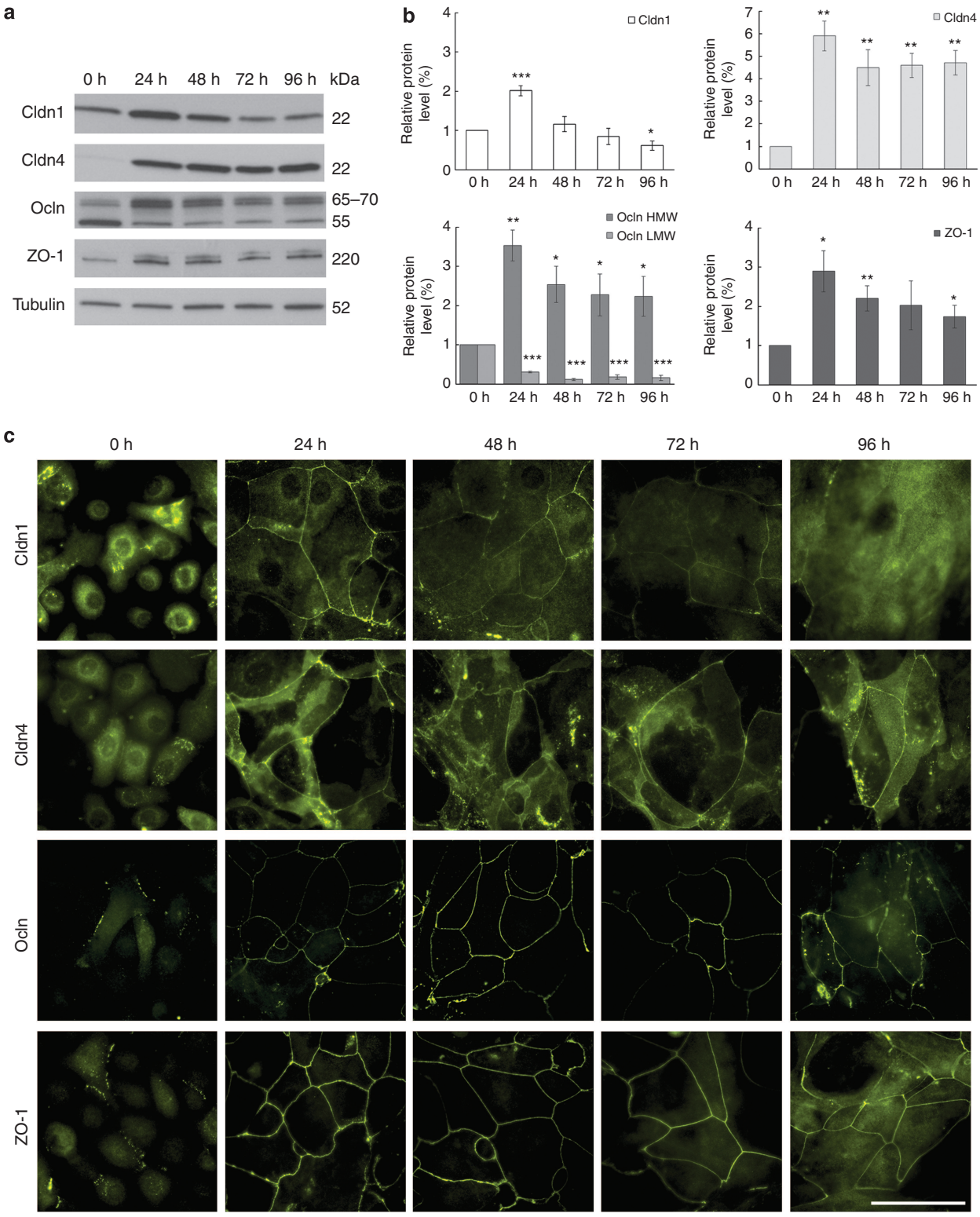

Figure 2. Protein levels and localization of tight junction (TJ) proteins in cultured human keratinocytes after Ca^{2+} switch. (**a**) Western blot of claudin-1 (Cldn1), claudin-4 (Cldn4), occludin (Ocln), and zonula occludens (ZO)-1 after Ca^{2+} switch. Equal amounts of proteins were separated and tubulin was used as gel-loading control. A representative experiment is shown ($n=3$). (**b**) Semiquantitative analysis of Cldn1, Cldn4, Ocln, and ZO-1. Band intensities were normalized to tubulin. Subsequently, the values were normalized to 0 hours ($n=3$; mean ± SEM; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with 0 hours). (**c**) Immunofluorescence localization of Cldn1, Cldn4, Ocln, and ZO-1 in cultured human keratinocytes at the indicated time points after Ca^{2+} switch. Bar = 20 μm.

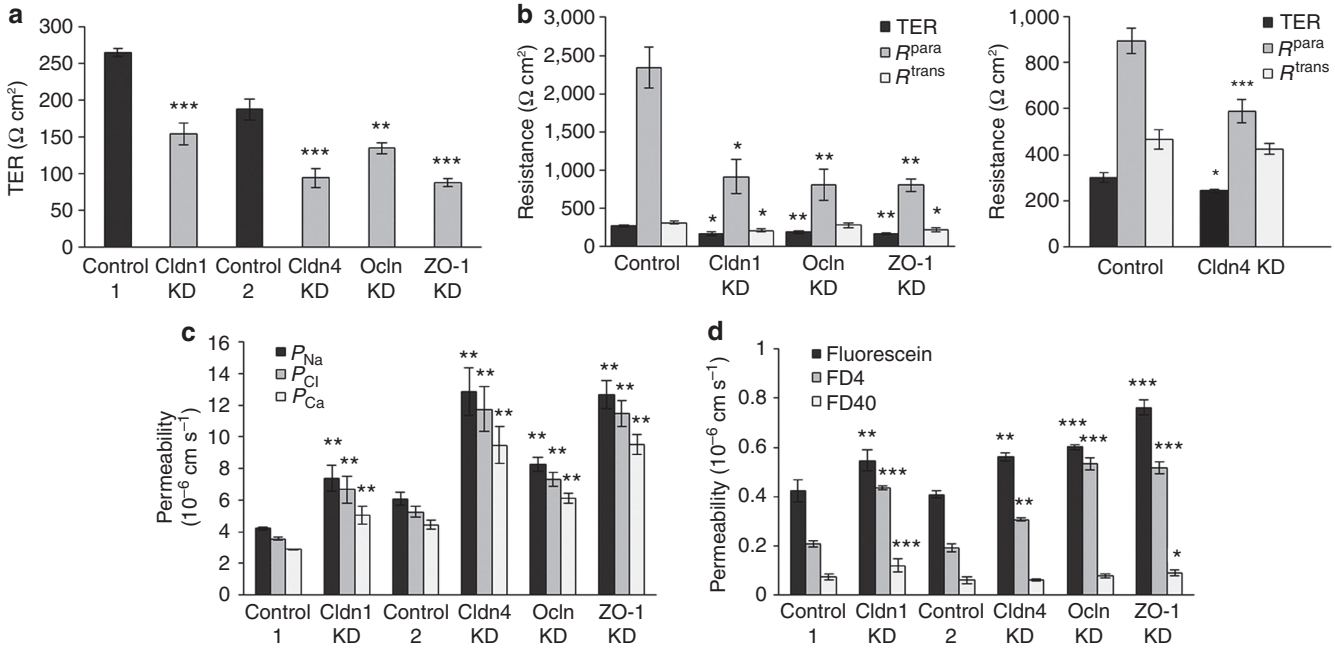

Figure 3. Effect of claudin-1 (Cldn1), claudin-4 (Cldn4), occludin (Ocln), and zonula occludens (ZO-1) knockdown (KD) on permeability for ions and molecules. (**a**) Transepithelial resistance (TER) of the indicated KD cells ($n = 5–8$). (**b**) Two-path impedance spectroscopy of R^{para}, R^{trans}, and TER in the indicated KD cells ($n = 3–8$). (**c**) Paracellular permeability for Na^+, Cl^-, and Ca^{2+} in KD cells ($n = 5–8$). (**d**) Tracer permeability assay for fluorescein, 4 kDa FITC-dextran (FD4), and 40 kDa FITC-dextran (FD40) in KD cells ($n = 6$). Owing to a limitation of cells, not all KD experiments could be performed with the same primary keratinocytes. Therefore, different controls are shown. All measurements were performed 48 hours after Ca^{2+} switch (mean ± SEM; $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with controls). Representative examples of experiments with individual cell lines are shown.

Another hypothesis that could explain an increase of transepidermal water loss without a direct loss of TJ water barrier would be an influence of Cldn1 KD on stratum corneum (SC) differentiation, and therefore on the SC water barrier. Therefore, we investigated the effect of Cldn1 KD on the protein levels of involucrin and transglutaminase1, proteins important for the formation of the cornified envelope (Proksch *et al.*, 2008). We observed a clear increase of protein levels for transglutaminase1 at all time points and for involucrin at certain time points (Figure 5d,e). In addition, we also found an upregulation of mRNA levels of loricrin (data not shown). These findings demonstrate that KD of Cldn1 can influence the expression of proteins forming the cornified envelope, even in submerged cultures.

DISCUSSION

In this study, we show that TJs in keratinocytes form a barrier to Na^+, Cl^-, and Ca^{2+}, and that increased TER in keratinocytes after Ca^{2+} switch mainly reflects reduced paracellular permeability for ions. We confirm that keratinocyte TJs form a barrier for intermediate-sized molecules and macromolecules. In addition, we demonstrate that KD of Cldn1, Cldn4, Ocln, and ZO-1 reduces R^{para} and increases permeability for Na^+, Cl^-, and Ca^{2+}, as well as for larger molecules, indicating that all of these proteins are critical for the formation of functional TJs in keratinocytes. Furthermore, we demonstrate that TJs in keratinocytes form a barrier to water, but that surprisingly KD of Cldn1 did not impair this barrier function. However, we did find that Cldn1 KD in submerged keratinocyte cultures influences proteins important for SC barrier formation.

Human and mouse keratinocytes have been shown to establish a TER, reflecting reduced permeability for ions, after induction of TJ formation by increased Ca^{2+} levels (Helfrich *et al.*, 2006; Yuki *et al.*, 2007). TER, however, is composed of both paracellular and transcellular components, and changes in ion permeability could reflect alterations in either ion transport between epithelial cells or across the plasma membrane (Krug *et al.*, 2009). Utilizing the two-path impedance spectroscopy, we show that the increase in TER is mainly caused by reduced paracellular ion permeability with only a small contribution by transcellular resistance.

Paracellular permeability consists of at least two functionally distinct permeability pathways through TJs: one that allows passage of ions and small uncharged molecules (pore or ion pathway) and one that allows also the flux of larger molecules (intermediate-sized molecules and macromolecules), but is not ion selective (leak pathway; Watson *et al.*, 2001; Van Itallie *et al.*, 2008; Shen *et al.*, 2011). We addressed both pathways here and show that maximal barrier function for intermediate-sized molecules and macromolecules (fluorescein, FD4, and FD40) is achieved 24 hours after Ca^{2+} switch, whereas for the ion pathway this is not the case until 48 hours. Upon reaching maximal barrier function, permeability for ions and fluorescein slowly increases until reaching steady state, whereas permeability for macromolecules remains stable.

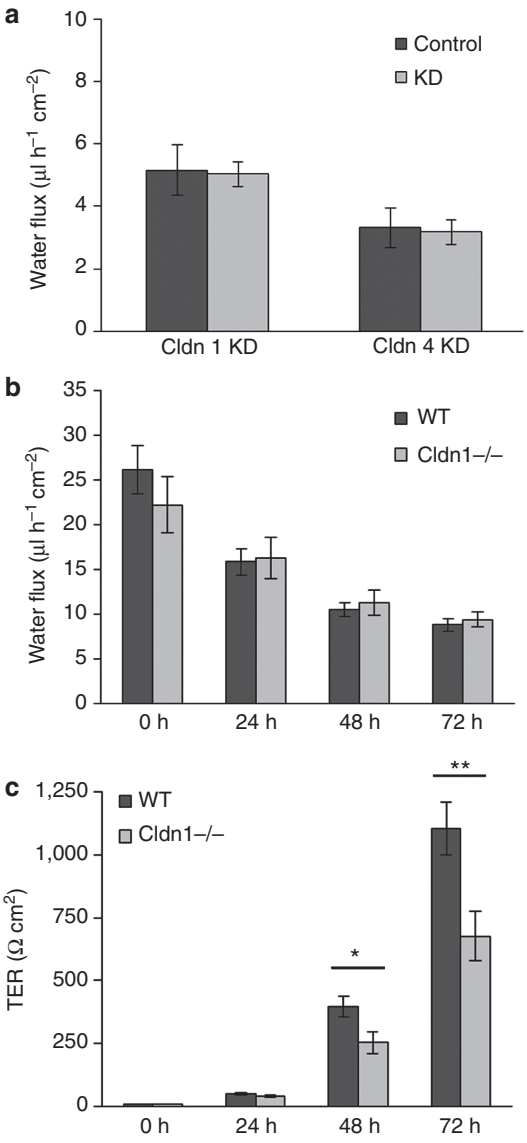

Figure 4. Water flux in claudin-1 (Cldn1)- and claudin-4 (Cldn4)-deficient cells. (**a**) Water flux measurements in human Cldn1 and Cldn4 knockdown (KD) keratinocytes performed 48 hours after Ca^{2+} switch ($n=4$; mean ± SEM). Representative examples of experiments with individual cell lines are shown. (**b**) Water flux measured in Cldn1-deficient (Cldn1 −/−) and wild-type (WT) mouse keratinocytes after Ca^{2+} switch ($n=3$; mean ± SEM). (**c**) Transepithelial resistance (TER) measured in Cldn1 −/− and WT cells at 0–72 hours after Ca^{2+} switch ($n=4$; mean ± SEM; $*P<0.05$, $**P<0.01$).

Maximal barrier function for intermediate-sized molecules and macromolecules at 24 hours corresponds with the highest TJ protein levels and their localization at the cell–cell borders. This suggests that the levels of Cldn1, Cldn4, Ocln, and ZO-1 are important to form the barrier for these molecules. This is additionally supported by our KD experiments, in which KD of each TJ protein resulted in a decrease of barrier function for fluorescein and FD4. These findings are in line with studies demonstrating that a functional TJ barrier for a 557-Da tracer is found only in the stratum granulosum, which is the layer of the epidermis where all TJ proteins colocalize (Furuse *et al.*, 2002; Kirschner *et al.*, 2010b). Interestingly, a shift in colocalization of the TJ proteins to deeper layers of the epidermis in psoriatic lesions results in a relocation of the barrier for the 557-Da tracer to these layers (Kirschner *et al.*, 2010b). De Benedetto *et al.* (2011) described a reduced expression of Cldn1 in nonlesional skin of patients with atopic dermatitis, hypothesizing that this could result in increased accessibility of the skin for allergens. A similar mechanism was suggested for the development of a progressive inflammatory skin disease in fibroblast growth factor receptor–deficient mice (Yang *et al.*, 2010). Our data strongly support these hypotheses, as we demonstrate that KD of Cldn1 increases TJ permeability for 4 and 40 kDa FITC-dextrans, a molecular weight range typical for allergens (King *et al.*, 1994).

Surprisingly, the strongest barrier for the pore pathway (ions) is observed at 48 hours, even though protein levels are decreased compared with 24 hours, and localization of Cldn1 at the cell–cell borders is reduced. This suggests that additional modifications of the TJ proteins are required to achieve maximal ion barrier formation, which might not be necessary for the formation of a barrier for larger molecules. It has been shown that phosphorylation of TJ proteins is important for the development of TJs (Helfrich *et al.*, 2006; Aono and Hirai, 2008; Raleigh *et al.*, 2011). However, we found that Ocln has the highest level of phosphorylation already at 24 hours (Figure 2). Therefore, we hypothesize that Ocln phosphorylation is more important for its targeting to the TJ than for ion permeability. In addition, Cldn4 phosphorylation does not seem to be a required event, as we found no change over time (data not shown). An analysis of additional TJ proteins to clarify this complex topic will be of interest.

With regard to the selectivity for ions, we find that there is not a strong preference for anions or cations. In addition, KD of the various TJ proteins resulted in an increased permeability for Na^+, Cl^-, and Ca^{2+} without changing the permeability ratio. This confirms data for Cldn1 in simple epithelial cells, showing that it clearly belongs to the tightening Cldns, which seal against larger molecules and without selectivity against ions (Inai *et al.*, 1999). The role of Cldn4, on the other hand, is inconsistent and, at least in part, depends on the molecular composition of the TJs and Cldn4 phosphorylation (Günzel and Fromm, 2012). Here we show that in keratinocytes Cldn4 has a barrier-forming role, sealing the TJs against ions independent of the charge.

Of special interest is the finding that TJs in keratinocytes form a barrier for Ca^{2+} and that this barrier can be impaired by the KD of Cldn1, Cldn4, Ocln, or ZO-1. There is a Ca^{2+} gradient in the epidermis with peak levels at the stratum granulosum and a steep decrease in the SC (Behne and Jensen, 2012), and our data suggest that these TJ proteins may contribute to this gradient. This result also supports the findings of Kurasawa *et al.* (2011) who observed increased Ca^{2+} localization in the SC after treatment of skin equivalents with the nonspecific TJ-opener sodium caprate, demonstrating that disruption of TJs in keratinocytes influences Ca^{2+} barrier formation.

Strikingly, even though we show here that TJs form a barrier for water in submerged keratinocyte cultures, we found that

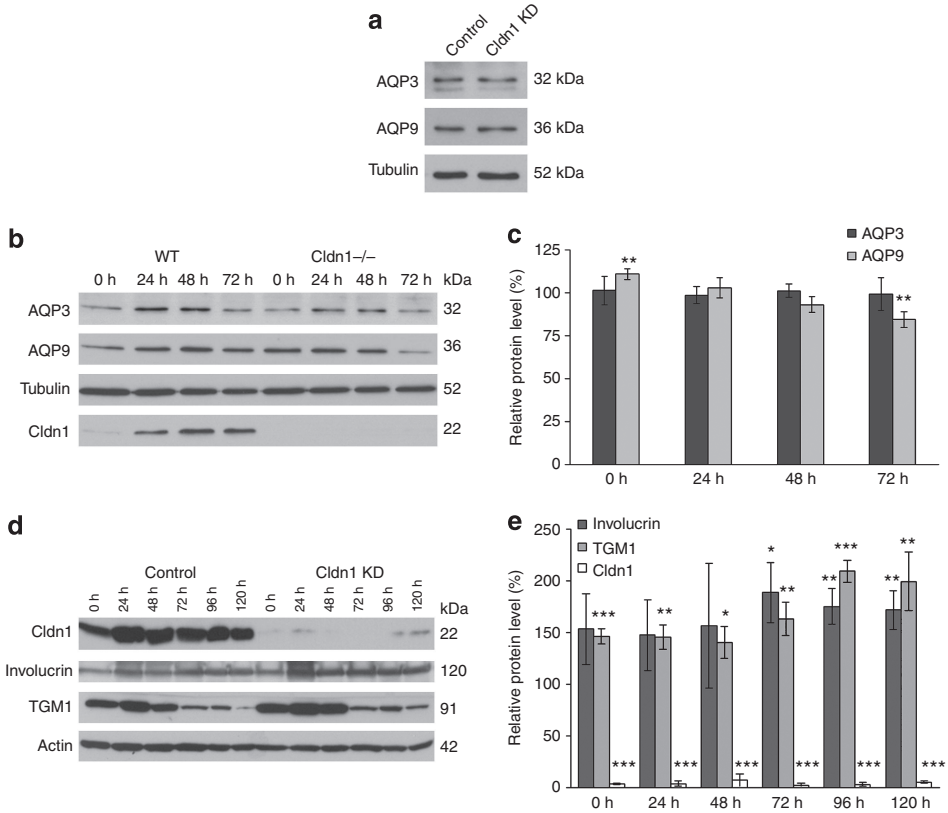

Figure 5. Aquaporins and cornified envelope proteins in claudin-1 (Cldn1) knockdown (KD) keratinocytes. Western blot of AQP3 and AQP9 (**a**) in control and Cldn1 KD cells 48 hours after Ca^{2+} switch ($n=3$) and (**b**) in Cldn1 −/− and wild-type (WT) cells at indicated time points ($n=3$). (**c**) Semiquantitative analysis; values of normalized protein band intensities of Cldn1 −/− were normalized to WT cells ($n=3$; $^{**}P<0.01$). (**d**) Western blot analysis of Cldn1, involucrin, and transglutaminase1 (TGM1) in control and Cldn1 KD cells at indicated time points after Ca^{2+} switch ($n=3$). (**e**) Semiquantitative analysis; values of normalized protein band intensities of Cldn1 KD were normalized to control cells ($n=3$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Examples of representative western blots are shown.

Cldn1 is dispensable for TJ water barrier function, in human and in mouse keratinocytes. This is surprising because Cldn1-deficient mice die within 1 day of birth because of elevated water loss (Furuse *et al.*, 2002). We investigated whether changes in AQP3 and AQP9, the main AQPs in the epidermis (Brandner, 2012), or in other TJ proteins may compensate for the loss of Cldn1 in cultured keratinocytes, but did not find a regulation of these proteins that could explain the lack of alterations in water flux. However, we cannot exclude that additional AQPs or other TJ proteins may compensate. In addition, we cannot exclude that the influence of Cldn1 in the epidermis might be more substantial. However, our results strongly suggest that Cldn1-dependent increased TJ water permeability is not responsible for the phenotype of the knockout mice. The main barrier for water in the epidermis is the SC, and it has already been suggested that the SC and the TJ barrier can influence each other (Michels *et al.*, 2009; Kirschner *et al.*, 2012). Indeed, even though our cell culture model is not equivalent to a fully functional epidermis *in situ*, we can already show that there is an alteration of proteins important for cornified envelope formation after Cldn1 KD. These proteins have been shown to be increased in skin with impaired barrier function, e.g., in psoriasis and Netherton syndrome (Bernard *et al.*, 1986; Raghunath *et al.*, 2004). Whether our observed upregulation of these proteins is a compensating effect, or whether it contributes to impaired water barrier function remains to be clarified. In addition, Cldn1 KD may also derange the lipid composition and orientation of the SC, which are also important for the SC barrier (Bouwstra and Ponec, 2006). Of note, in human patients with a complete loss of Cldn1 (neonatal ichthyosis–sclerosing cholangitis syndrome), a much less severe phenotype than in Cldn1-deficient mice is observed (Hadj-Rabia *et al.*, 2004; Feldmeyer *et al.*, 2006), hinting for the major defect in the SC, which can be compensated over time in humans. In addition, the epidermis of Cldn1-deficient mice show a more compact SC, even though it is leaky for water (Furuse *et al.*, 2002).

The elucidation of the mechanisms for how alterations of Cldn1 influence cornified envelope proteins and lipids in submerged cultures and also in skin equivalents will be a challenging task in the future.

Overall, our results demonstrate that barriers for diverse ions and molecules of different size are formed in keratinocytes by TJs, and that the TJ proteins Cldn1, Cldn4, Ocln, and ZO-1 contribute to these barriers. TJs also form a barrier for water,

but Cldn1 and Cldn4 are dispensable for this barrier; however, Cldn1 influences important components of the SC barrier.

MATERIALS AND METHODS

Cell culture

Primary human keratinocytes were isolated from foreskin as described (Moll *et al.*, 1998) and cultured in serum-free keratinocyte growth medium (KGM2, PromoCell, Heidelberg, Germany). Cldn1-deficient mice were generated as described (Furuse *et al.*, 2002), and keratinocytes were isolated from newborn mice as previously described (Helfrich *et al.*, 2006). For the investigation of barrier properties, keratinocytes were seeded on porous culture plate inserts (Millipore, Cork, Ireland; 0.4-μm pore size), transferred to high-calcium-containing medium (1.8 mM Ca^{2+}, Ca^{2+} switch; Quantum 286, PAA, Pasching, Austria), and cultured submerged for the indicated time periods.

Antibodies, primers, and siRNA

Antibodies specific for Cldn1 (71-7800), Cldn4 (18-7341), Ocln (mouse: 33-1500; rabbit: 71-1500), and ZO-1 (33-9100) were purchased from Life Technologies (Darmstadt, Germany). Antibodies for tubulin (CP06) were from Calbiochem (Darmstadt, Germany); AQP3 (sc-9885) and AQP9 (sc-74409) from Santa Cruz Biotechnology (Santa Cruz, CA); involucrin (NCL-INV) from Leica Biosystems (Newcastle, UK); transglutaminase1 (ABIN576039) from Antibodies-online (Aachen, Germany); and actin (AC-15) from Sigma (Munich, Germany).

FAM dye-labeled real-time PCR (qRT–PCR) TaqMan MGB probes for Cldn1 (Hs00221623_m1), Cldn4 (Hs00533616_s1), Ocln (Hs00170162_m1), ZO-1 (Hs01551861_m1), loricrin (Hs01894962_s1), and GAPDH (Hs03929097_g1) were purchased from Applied Biosystems (Carlsbad, CA).

Ready-to-use siRNAs for human Cldn1 (SI04279114 and SI03206336), Cldn4 (SI03064418 and SI00025207), Ocln (SI03225999), ZO-1 (SI02655149), and control siRNA (1027280) were purchased from QIAGEN (Hilden, Germany).

siRNA experiments

Third-passage primary human keratinocytes were transfected by using HiPerFect Transfection reagent (QIAGEN) according to the manufacturer's instructions. For details, see Supplementary Methods online.

Western blot analysis

Lysis of cultured keratinocytes in RIPA buffer and western blot analysis were performed as described (Ohnemus *et al.*, 2008). Antibody dilutions were as follows: Cldn1: 1:2,000; Cldn4: 1:200; Ocln (rabbit): 1:3,000; ZO-1: 1:200; AQP3: 1:100; AQP9: 1:100; Inv: 1:5,000; transglutaminase1: 1:500; Tubulin: 1:2,000; Actin: 1:10,000. Signal intensities were densitometrically quantified with ImageJ 1.43u software (National Institutes of Health, Bethesda, MD).

Tracer permeability assays

Tracer permeability assays were performed as described (Kirschner *et al.*, 2011). For details, see Supplementary Methods online.

Immunofluorescence microscopy

Cultured cells were fixed and stained as previously described (Kirschner *et al.*, 2009). All antibodies were used in a 1:100 dilution. Isotype-matched antibodies were used as negative controls. All images of stainings from a series of experiments were acquired and processed with the same settings and representative areas were photographed.

Electrophysiology

Electrophysiology investigations were carried out in Ussing chambers specially designed for the investigation of cell monolayers grown on cell filters (Kreusel *et al.*, 1991). Transepithelial voltage and TER were measured in Ussing chambers after correction of the values for empty filters and the bathing solution. Determination and calculation of R^{trans}, R^{para}, and TER were performed as described (Krug *et al.*, 2009). Permeabilities for Na^+, Cl^-, and Ca^{2+} were determined from bi-ionic potential measurements and calculated using the Goldman–Hodgkin–Katz equation as previously specified (Günzel *et al.*, 2009). A prerequisite for bi-ionic potential measurement and calculation of permeability is the existence of TJs. Before Ca^{2+} switch at 0 hours, TJs were not formed, and thus these permeability measurements could not be performed. Therefore, no value at 0 hours exists.

Measurement of transepithelial water flux

The measurement of transepithelial water flux was performed in a modified Ussing chamber as described in detail by Rosenthal *et al.* (2010). Briefly, cell filters were mounted into the Ussing chambers and maintained in HEPES-buffered Ringer's solution at 37 °C under constant circulation. The transepithelial voltage was clamped to 0 mV to avoid any effects on ion and water flux. Water flux was induced by a transepithelial osmotic gradient with mannitol (100 mM) and the fluid level in both chambers was monitored by a video optic system ColorView XS (Olympus Soft Imaging Solutions GmbH, Muenster, Germany), at time 0 minutes and every 10 minutes over a period of 100 minutes. Water flux was calculated from the difference between the menisci at the registration times after calibration of the system.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using Student's *t*-test and the Bonferroni–Holm correction for multiple comparisons. Significance levels are denoted: $*P<0.05$, $**P<0.01$, $***P<0.001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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